academicJournals

Vol. 12(16), pp. 1915-1920, 17 April, 2013
DOI: 10.5897/AJB11.3254
ISSN 1684–5315 © 2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Optimization of chemiluminescent detection of mitochondrial RNA-protein interaction by nonradioactive mobility shift

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Accepted 27 March, 2013

RNA mobility shift is one among many procedures used to study RNA-protein interaction. Yet, there are some limitations for the radioactive RNA mobility shift including; 1) the risk of using radiolabeled nucleotides, 2) the long time to get the results; this could range from days to weeks, and 3) its high cost as compared to nonradioactive techniques. In this study, we optimized a nonradioactive procedure using dig-11-UTP nucleotide and chemiluminescent detection for mitochondrial RNA-protein interaction. The optimizations include the quality limiting steps such as using non-specific competitors of RNA probe, UV-cross linking time, electrotransfer to membranes, application with various protein extracts, and the examination for false positive RNA-protein complexes using proteinase K digestion. The results show that the optimizations carried out in the study significantly enhanced the quality of the results obtained with this procedure.

Key words: RNA mobility shift, mitochondria, wheat, RNA-protein interaction, chemiluminescent detection, nonradioactive.

INTRODUCTION

Genetic information stored in DNA is converted into biological data in protein sequences through the two complex processes: transcription and translation. RNA binding proteins play an important role in these two and other related biological processes such as splicing and RNA editing (Mulligan et al., 1992; Lavigueur et al., 1993; Suzuki et al., 2010). The interface of RNA-binding proteins is a wide stage with many interconnected players that organize and control the final image of gene expression. RNA mobility shift assay is a vital method for studying RNA-protein interaction which is used in studying the interplay between RNA sequences and protein factors to: 1) modify the structure of RNA molecules (Li et al., 1999; Ficzycz and Ovsenek, 2002); 2) regulate gene expression at the posttranscriptional and translational levels (Le et al., 1997; Mochow-Grundy et al., 2001; Wei et al., 2001; Randall et al., 2002); 3) determine the essential *cis acting* sequences in RNA molecules (Ruth et al., 1999; Berkowitz et al., 1993; Dixon et al., 2000; Totary-Jain et al., 2005; Crooks et al., 2007); 4) study the structure/function relationship of secondary structure domains of RNA molecules (Blyn, 1996; Le et al., 1997; Shen et al., 2001); 5) study the minimum essential parts for RNA-protein binding (Berkowitz et al., 1993; Graff et al., 1998; Jonas et al., 2008) and 6) study the

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role of 3' and 5' untranslated regions of mRNA (Chang et al., 1994; Le et al., 1997; Thekkumkara and Linas, 2003; Totary-Jain et al., 2005; Jonas, 2008). The technique is widely applied in various array of living organisms including animal and animal cell cultures (Ficzycz and Ovsenek, 2002; Totary-Jain et al., 2005; Crooks et al., 2007; Suzuki et al., 2010), plant cells (Shen et al., 2001), bacteria (Wei et al., 2001; Jonas et al., 2008) and viruses (Graff et al., 1998; Kim et al., 1998; Li et al., 1999). Mobility shift assay is one of the predominant procedures that are used in analysis of RNA-protein interaction (Thomson et al., 1999) because of many reasons including its higher sensitivity and lake of reliable reproducible alternatives.

Nonradioactive methods have been developed to replace the radioactive ones. They have some advantages over the radioactive labeling methods. They have shorter exposure time which means getting faster results of an experiment. In addition, they save time and money because of the long half life of labeling molecules and they offer homogenous labeling with minimum number of probe preparation. More importantly, they are safer because they do not include exposure to radioactive molecules. Nonradioactive labeling using Digoxigenin-11-UTP along with chemiluminescent detection is among nonradioactive methods which were introduced to replace the radioactive procedures (Vaquero et al., 1998).

Although, the method has all advantages of nonradioactive methods, yet there has not been enough optimization for the protocol to enhance its efficiency. In this report, we have made some optimizations for the key steps which determine the quality of the results obtained by this protocol using wheat mitochondrial protein extracts and *orf256-coxI* RNA.

MATERIALS AND METHODS

Mitochondrial proteins

Mitochondrial protein extracts of four wheat lines [cytoplasmic male sterile (CMS); *Triticum aestivum* (Ta); fertility restored (FR1); *Triticum temopheevi* (Tt)] were prepared according to a published method (Song and Hedgcoth, 1994).

Electrotransfer of proteins to PVDF membrane

Proteins were blotted to a PVDF membrane (Immobilon-P, Millipore) using a BioRad semi-dry electrobloter for 1 h at 2 mA/ cm^2 of PVDF membrane using the manufacturer instructions and buffers (Millipore, USA; El-Shehawi et al., 2003).

RNA probe

RNA probe was prepared using *in vitro* transcription from the mitochondrial gene *orf256-coxI* (Rathburn and Hedgcoth, 1991) using *in vitro* transcription kit according to the manufacturer instructions (Epecentre, USA) with replacement of the ^{32}P-UTP with DIG-11-UTP. The ratio of UTP : DIG-11-UTP in the *in vitro* transcription reaction was 0.65:0.35 as recommended by the manufacturer (Roche). The RNA probe was precipitated after transcription according to the manufacturer's instructions (Roche).

RNA mobility shift

Mobility shift assays were done by modifications of a nonradioactive method (Berger et al., 1993). Mitochondrial protein extracts (20 µg), were incubated in a 20 µL reaction containing 40 U RNase inhibitor in mobility shift assay buffer (10 mM Tris-HCl, pH 7.5; 50 mM NaCl; 1 mM MgCl; 0.5 mM EDTA; 4% glycerol; 0.5 mM DTT) for 5 min at room temperature. Yeast RNA (4 µg), was added (Kafert et al., 1998) and the reaction was incubated for 10 min. RNA probe was added and incubation was continued for 30 min at room temperature. The reaction mixture was UV cross-linked for 10 min at 254 nm at 5 cm from the UV source (Zehner et al., 1997). Unprotected RNA probe was removed by the addition of 1 µL of RNase cocktail (Ambion, USA) and incubation for 15 min at 37°C. Following SDS-PAGE (Laemmli, 1970), separated components were electrotransfered to PVDF membrane. The membrane was heated for 1 h at 80°C to fix RNA to the membrane.

The blot was blocked by three changes of 30 min each of blocking buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 10% glycerol; 2.5% Nonidet P-40; 0.1 mM DTT; 5% casein) and rinsed in detection buffer (50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 0.25% casein, USB). The blot was incubated in anti-digoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim) solution, diluted (1:10,000) in detection buffer for 30 min with gentle shaking at room temperature. The blot was washed four times for 10 min each time with gentle shaking in detection buffer and once in assay buffer (0.1 M diethanolamine, pH 10; 1 mM $MgCl_2$; 0.02% sodium azide) (Tropix). CDP-star was applied (2.5 µL of 1:100 diluted CDP-Star in assay buffer per cm^2 of membrane). The blot was exposed to X-ray film for 30 min to 1 h.

RESULTS AND DISCUSSION

Nonspecific competitor

Yeast RNA and heparin are usually used as nonspecific competitors in mobility shift assays. Yeast RNA is added before the probe to block nonspecific sites, whereas heparin is added after binding the probe to its sites to remove the nonspecific binding. Various concentrations of yeast RNA as well as heparin were tested with only Ta extract (Figure 1). Heparin seems to give faint diffused bands at lower concentrations (2.5 and 5 µg/µL). These bands became undetectable at 10 µg/µL. The same bands detected using yeast RNA was sharper and more intensed (Figure 1), therefore yeast RNA serves as a better nonspecific competitor than heparin.

UV cross-linking time

Time of UV cross-linking is a sensitive factor for stabilizing the RNA probe to its target protein factor especially if binding is not stable on native gels. The exposure time of RNA-protein complexes to the UV is a limiting factor. Overexposure to UV could lead to cross-linking of the probe molecules that can be detected as bands giving misleading results. Underexposure to the UV could result in missing some proteins which may be important.

UV cross-linking times ranging from 2 to 15 min were tried. The intensity of RNA-protein bands increased with

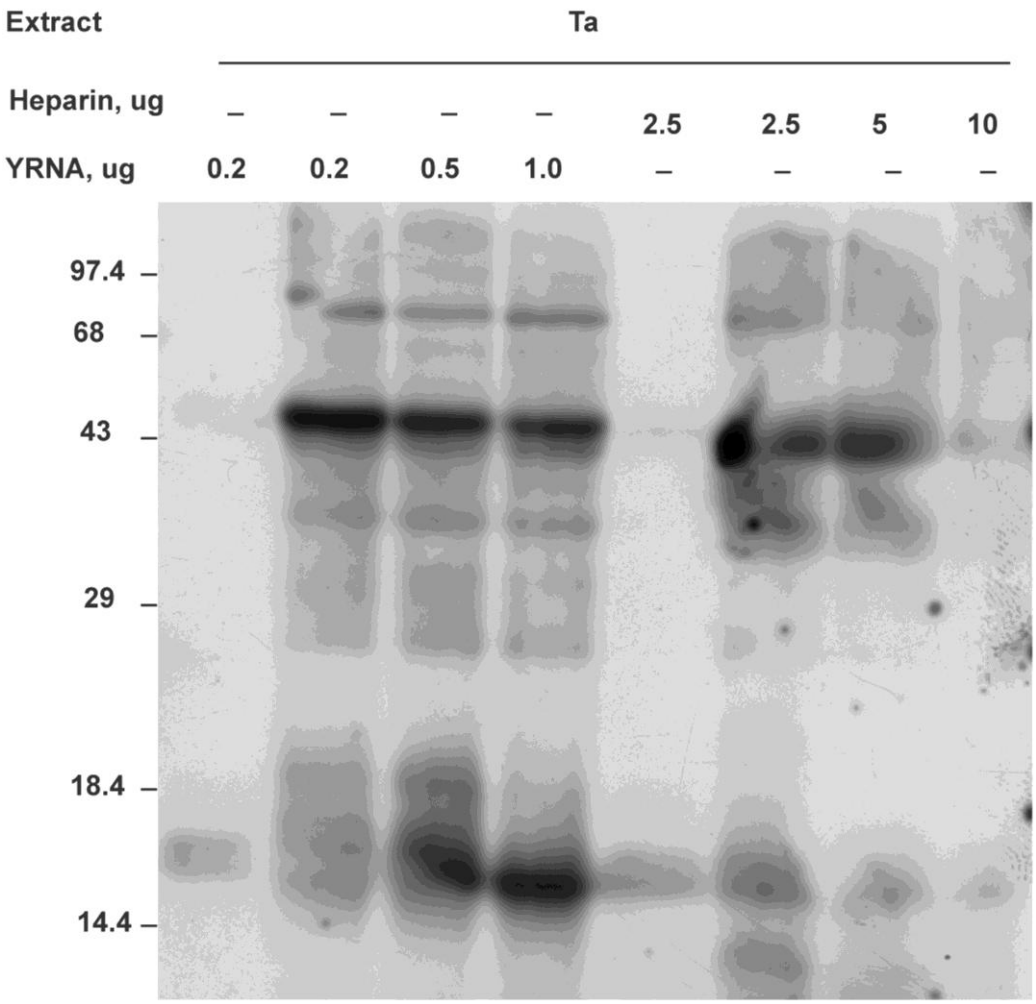

Figure 1. Effect of non-specific competitors on the formation of mitochondrial RNA-protein complexes. Mitochondrial protein extract (20 µg) was incubated with DIG-labeled *orf256-coxI* RNA probe in the presence of increased concentration of yeast RNA or heparin. For yeast RNA, 0.2 (lane 1, 2), 0.5, (lane 3) and 1.0 (lane 4) µg/µL were used, whereas, 2.5 (lane 5, 6), 5 (lane 7) and 10 µg/µL (lane 8) of heparin were used. Lane 1 and 5 do not contain any protein extract and served as negative controls.

increasing time of UV cross-linking (Figure 2A). It appeared that 10 min of UV cross-linking is an optimum time under these conditions since it showed a reasonable intensity of the 46 kD band and minimal intensity of the 16 kD bands which may be due to UV cross-linking of probe fragments.

Electrotransfer to PVDF membrane

Electrotransfer is an essential determinant of the nonradioactive mobility shift because if it is not done appropriately, could lead to missing of the RNA-protein complexes. During electrotransfer, double PVDF membranes were used to test if some proteins leak through the first membrane. No bands were detected on the second membrane indicating that there is no protein leakage through the first membrane (Figure 2B).

Also, the gel was stained after electrotransfer to make sure there was no significant amount of proteins retained in the gel. It seems that there was no RNA-protein complexes transferred to the second membrane. Staining the gel also indicated that most of the proteins were transferred to the PVDF membrane.

Using of different protein extracts

To confirm that this procedure can be used with different sources of mitochondrial proteins, another three wheat mitochondrial extracts (CMS, FR1, Tt) were tested against the *orf256-coxI* probe under the same conditions. Five protein bands were detected between 200 and 68 kD, some

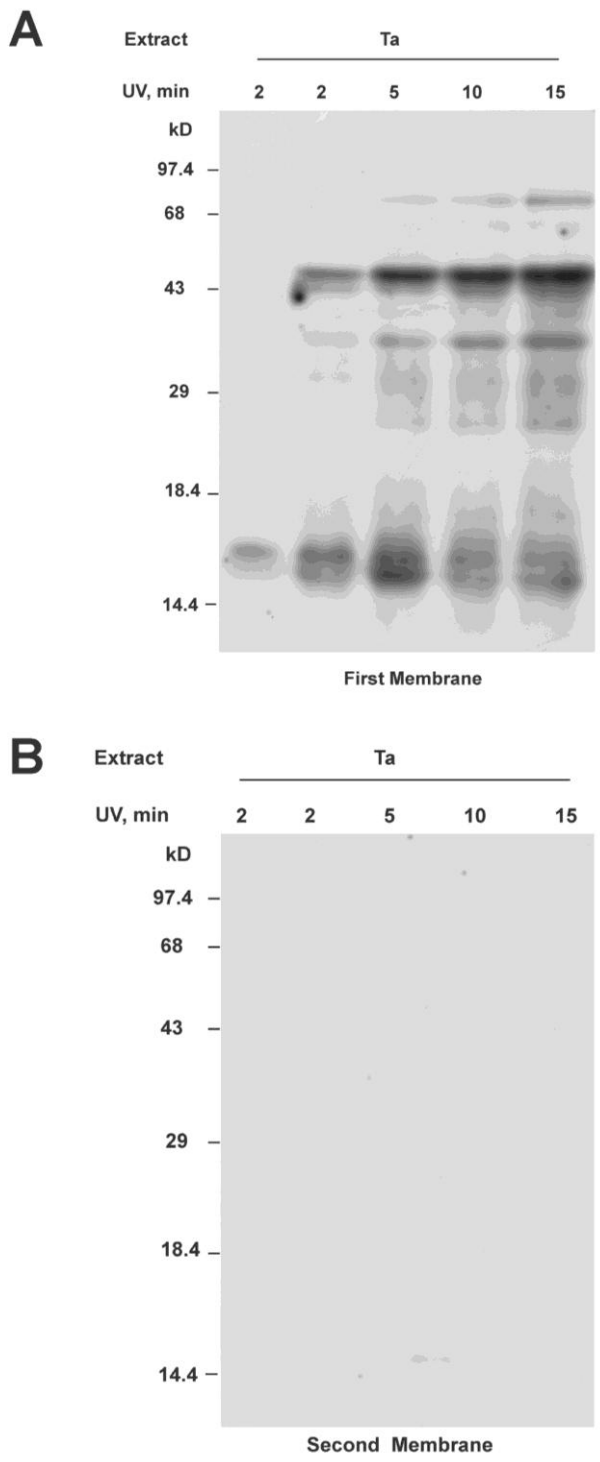

Figure 2. Effect of UV cross-linking and electrotransfer time on the formation (a) of and transfer (b) of mitochondrial RNA-protein complexes. Ta mitochondrial protein extracts (20 µg) was incubated with *orf256-coxI* RNA probe in the presence of 0.2 µg/µL yeast RNA. After RNA-Protein binding, samples were subjected to 254 nm UV light for 2 (lane 1, 2), 5 (lane 3), 10 (lane 4) or 15 (lane 5) min on ice at 5 cm from the UV source. Double PVDF membrane was used during electrotransfer (A, B) to test if some proteins leaked through the first membrane. The rest of the protocol is done as stated in the text.

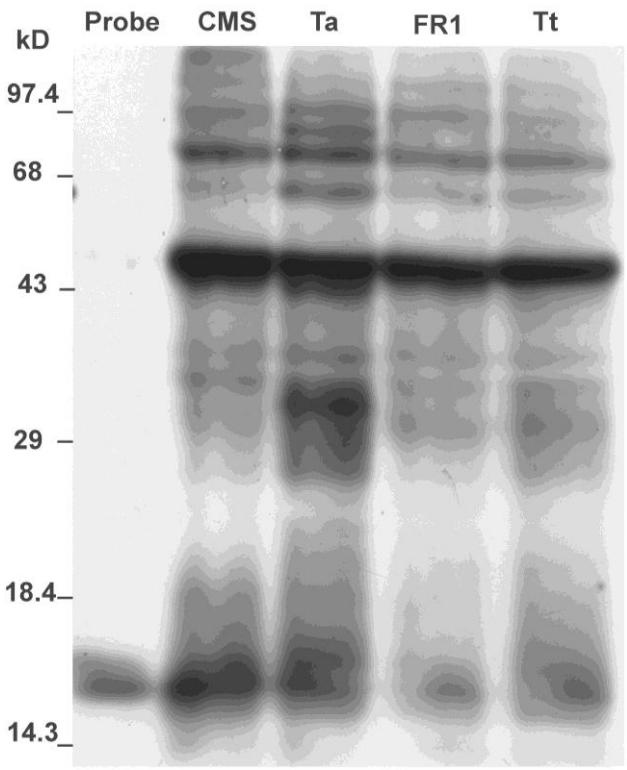

Figure 3. Optimization of RNA-protein interaction with various mitochondrial protein extracts. Different mitochondrial protein extracts were incubated with *orf256-coxI* RNA probe under the above conditions. Lane 2, CMS; lane 3, Ta; lane 4, FR1; lane 5, Tt. Lane 1 is negative control since it does not contain any protein extract.

of which differ in intensity, but all are detected in all extracts (Figure 3). One heavy band of about 46 kD and two bands of about 35 kD were also detected in all extracts.

There was one band at about 16 kD that was detected in all extracts and was present in the free probe lane as well. This band might represent UV cross-linked fragments of the RNA probe. Giving similar bands with various mitochondrial protein extracts confirm that this procedure is reproducible with different protein sources (Figure 3).

Proteinase K treatment

Since the bands detected on the mobility shift chemilumnigram may be detected from either real RNA-protein complexes or cross-linked fragments of the RNA probe, to distinguish between both possibilities, Proteinase K was used to digest proteins after UV cross-linking and before RNase treatment.

Proteinase K treatment abolished all bands detected under these conditions (Figure 4). Results obtained from this study provide an evidence that these optimizations contribute significantly in enhancing the quality of nonradioactive mobility shift assay to study mitochondrial

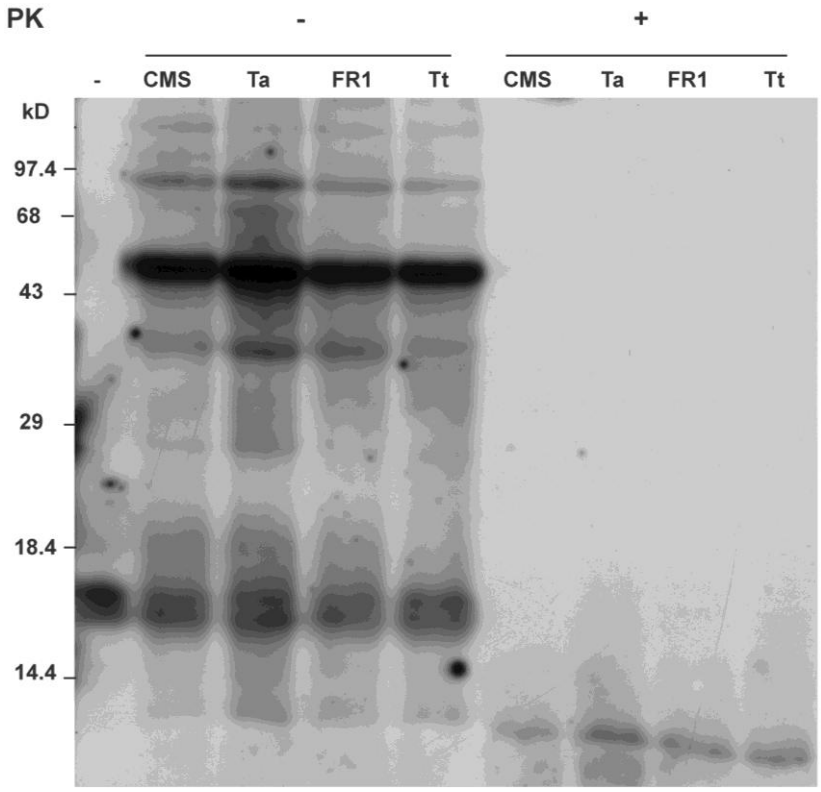

Figure 4. Differentiation of real RNA-protein complexes using proteinase K digestion. Mitochondrial proteins from various wheat lines (Figure 3 legend) were used in mobility shift reactions as explained in the text. Lanes 6, 7, 8 and 9 show identical reactions except that the reaction mixes were treated with Proteinase K (PK) for 10 min at 50°C. Proteinase K treatment was done after UV cross-linking and before RNase treatment.

plant RNA-protein interaction.

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